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MEMOIRS OF THE DEPARTMENT OF AGRICULTURE IN INDIA

A NEW NITRITE-FORMING ORGANISM

BY

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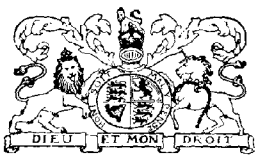
First Assistant to the Imperial Agricultural Bacteriologist



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A NEW NITRITE-FORMING ORGANISM.

BY

N. V. JOSHI, B.A., B.Sc., L. Ag.,

First Assistant to the Imperial Agricultural Bacteriologist.

THE following experiments were begun in the laboratory of the Imperial Agricultural Bacteriologist at Pusa with the object of demonstrating to students the method of isolation of nitrifying organisms.

The solution used had the usual ingredients of Omélianski solution, calcium carbonate being substituted for magnesium carbonate in order to avoid any inhibition of nitrification previously noticed in this laboratory.[1] The solution was used in its dilute form and had the following composition :—

Ammonium Sulphate	.. ..	0·5 gm.
$K_2H PO_4$	.. .. ..	0·25 ,,
$MgSO_4$ ..	.. .. ..	0·1 ,,
NaCl ..	.. .. ..	0·5 ,,
$FeSO_4$ ..	.. .. ..	0·1 ,,
Calcium Carbonate	.. .. ..	10·0 ,,
Distilled Water	.. .. ..	1000·0 c.c.

Pusa soil 1·0 gram was used as inoculum in 50 c.c. of solution. When nitrite formation had progressed sufficiently (as tested by napthylamine and sulphanilic acid) which took place in about a fortnight, 5 c.c. of the liquid was transferred to another flask containing 50 c.c. sterile solution of the same composition. When nitrite formation had taken place in the second flask 5 c.c. from this was transferred to a third flask.

After some six similar transfers a thin pellicle dotted with chalky white prominences and corresponding to the description of growth of *B. Oligo-carbophilous* was seen on the surface of the fluid and was at first thought to be due to the presence of this organism ; it was found, however, that entirely distinct morphological characters excluded this possibility. Microscopic examination showed the pellicle to consist of several kinds of bacteria among which was an organism which had the appearance of a mass of ramifying threads.

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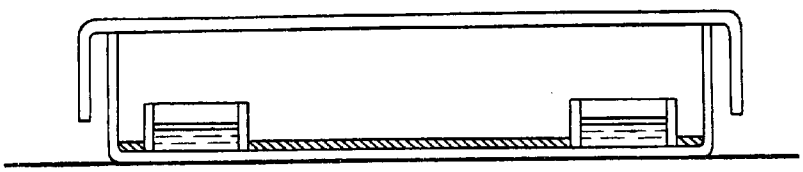
[1] Hutchinson, C.M. " Studies in Bacteriological Analysis of Indian Soils, No. 1, 1910-11." *Mem. Dept. of Agri., India, Bact. Ser.*, Vol. I, No. 1.

An attempt was made to plate the fluid culture on silicate jelly prepared according to Stevens and Temple's[1] method and on Beijerinck's agar as described in Erwin Smith's text-book.[2] A variation was introduced into the latter medium by substituting ammonium sulphate for hydrogen ammonium sodium phosphate (which is recommended as the best ammonium salt). In our experiments no such difference was noticed in the growth on the two kinds of agar prepared and latterly agar with ammonium sulphate was invariably used.

In order to increase the size of colonies of nitrifiers it is necessary to add some ammonium sulphate solution so as to be absorbed by the solid substratum. Omélianski[3] makes two cavities by cutting out small portions of the solid medium by means of a sterile knife and adds the solution into these cavities. This is inconvenient and there is a possibility of some of the solution spreading over the solid substratum. In our experiments, therefore, use was made of glass rings the top of which protruded over the medium as shown in the accompanying figure. The rings were sterilized along with the Petri



Petri-dish with glass rings for plating Nitrifying Organisms.

dishes, and silicate jelly or ammonium sulphate agar was poured in the plates, and, when required, sterile ammonium sulphate solution was put in the glass rings and was slowly absorbed by the medium.

No organism grew on either of the media for the first ten days or so, after which small chalky white colonies were seen (Plate I, Fig. 2). These, when examined under the microscope, resembled the mass of threads originally found. After addition of ammonium sulphate into the glass rings the size of the colonies increased up to about 0·5 cm. in diameter. One of the colonies was inoculated into 20 c.c. sterile dilute Omélianski solution in a test tube. After incubation at 30°C for about 15 days nitrites were found but no nitrates. Microscopic examination showed, besides a mass of ramifying threads, another organism with thread-like projections at each extremity, resembling flagella,

[1] *Centr. für Bakt.* II, Abt. 21 Bd., p. 84.
[2] Erwin Smith, "Bacteria in relation to plant diseases," Vol. I, p. 199.
[3] Omélianski, *Centr. für Bakt.* II, Abt. 5 Bd., p. 537.

which stained faintly with carbol fuchsin alone but quite distinctly by Zettnow's method. This culture was therefore at that time supposed to be impure and so an attempt to purify the culture was made by growing the organism again from fluid cultures by successive transfers to fresh flasks. After about six more transfers an attempt was made to plate on gypsum block[1] and filter paper[2] pad as recommended by Omélianski. On gypsum no colonies grew for a long time ; on filter paper, however, small brown colonies began to make their appearance in two days' time and grew rapidly. These colonies corresponded to the description of colonies of nitrite-former given by Omélianski. Microscopic examination, however, revealed the fact that in addition to bacteria of different kinds actively motile protozoa had appeared.

The appearance of protozoa, when filter paper was used to grow the colonies of this nitrite-former, is rather strange, especially after so many generations of the nitrite culture. The inoculum which was used, *viz.*, 5 c.c. would, however, be sufficient to carry over a few protozoa or cysts at each transference. The original solution in flasks used for inoculating Petri dishes with filter paper was examined for protozoa ; it showed only one or two in a hanging drop but nothing like the large number noticed while examining the brown colonies on filter paper. This suggested the possibility of the protozoa coming from the filter paper. Control plates however did not show any growth of the protozoa. Fresh plates were also made to satisfy ourselves that the filter paper was not responsible for the latter. None appeared on filter paper with Omélianski solution while large numbers were seen on filter paper incubated with nitrifying culture which they must have accompanied all along. The occurrence of protozoa with nitrifying cultures is not a new discovery. Beijerinck[3] found one kind of amœba accompanying nitrifiers. J. Killer[4] recently made use of Omélianski solution along with other synthetic media in order to grow protozoa by inoculating it with soil and then increasing the nitrifying organisms which would serve as a food to the protozoa. This investigator found only a few protozoa in the solution and therefore did not consider it as a useful medium for the purpose. His experiments with hay infusion, Giltay's solution, and Omélianski solution were therefore repeated by inoculating these solutions in three flasks with Pusa soil. These were not successful as very few protozoa appeared in the solution. Filter paper

[1] Omélianski, *Centr. für Bakt.* II, Abt. 5 Bd., 1899, p. 652.
[2] Do. Do. Do. Do. 8 Bd., 1902, p. 785.
[3] Beijerinck, *Centr. für Bakt.* I, Abt. 19 Bd., 1896, pp. 257-267.
[4] Killer, *Centr. für Bakt.* II, Abt. 37 Bd., 1913, p. 521.

pads were now prepared as before by moistening with distilled water and sterilizing them in Petri dishes at 130°C for half an hour. These were afterwards inoculated from three flasks respectively after separate addition of the necessary sterile solution to each of them.

In two or three days' time similar brown colonies appeared and spread over the whole surface of the filter paper pads. Few protozoa were seen in solution but quite large numbers were noticed on the filter paper. Giltay's solution did not prove as successful as hay infusion and Omélianski solution.

A. Cunningham and Löhnis[1], in a paper published recently, have communicated their observations on protozoa. They also used several media for growing protozoa and got good results with Omélianski solution. They found large numbers of flagellates and ciliates from ærobic cultures of cellulose organisms with $K_2H\ PO_4$ and $Mg\ NH_4\ PO_4$. The use of filter paper pads in our experiments also probably increases some cellulose organisms which serve as a better food for protozoa. This seems to be the probable explanation of the fact that large numbers appear when filter paper pads are used. Be that as it may, the use of filter paper with a shallow layer of nutritive solution appears to give better facilities for the growth of protozoa.

After some time the chalky white colonies also grew on filter paper. On gypsum block only chalky white colonies made their appearance after about 8 days. These on examination showed the ramifying threads accompanied by the flagellated organism. In order to separate these two kinds, as they were then supposed to be, recourse was had to pasteurisation. For this purpose some of the colonies on gypsum were emulsified in sterile distilled water. The emulsion was put into two tubes which were then heated to 40°C and 60°C respectively for half an hour, and then inoculated in dilute Omélianski solution. Tested as to whether nitrification had progressed, both of them gave reactions for nitrites but not for nitrates. Plated on filter paper pads the culture which had received the inoculum heated to 60°C showed chalky white colonies but no brown colonies. The colonies were grown subsequently on gypsum block, silicate jelly, ammonium sulphate agar, and unglazed porcelain dishes, and showed the same chalky white appearance. Examined under a low power of the microscope the colonies were seen to be opaque with a brownish centre and filamentous edges.

They were also grown on slants of silicate jelly and ammonium sulphate agar; the appearance of these streak cultures was chalky white with no precipitate in the condensation water. (Plate I, fig. 1.)

[1] A. Cunningham & Löhnis, *Centr. für Bakt.* II, Abt. 39 Bd., 1913-14, p. 596.

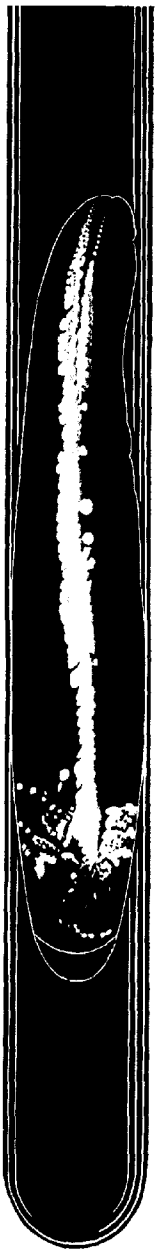


Fig. 1.

NITRITE FORMER

growth on
Ammonium Sulphate Agar.
Size $\times 1\frac{1}{2}$.

Fig. 2.

NITRITE FORMER

Colony on Ammonium Sulphate Agar.
Size $\times 55$.

Fig. 1.

NITRITE FORMER

Flagellated Gonidia.
Zettnow's method × 1300.

Fig. 2.

NITRITE FORMER

Stage showing Bi-polar staining.
Carb. Fuchsin × 1100.

Fig. 3.

NITRITE FORMER

Stage showing branching.
Carb. Fuchsin × 1000.

Fig. 4.

NITRITE FORMER

Thread form showing Sheath.
Archibald's Stain × 900.

Fig. 5.

NITRITE FORMER

Thread form showing Sheath.
Archibald's Stain × 1400.

When grown on plates of silicate jelly and ammonium sulphate agar in streaks they had the same chalky white appearance. The plates were examined for nitrites and nitrates. Nitrites were found but no nitrates.

Microscopic examination of organisms from colonies showed the ramifying threads and flagellated organisms mentioned above.

The organism when inoculated on ordinary agar and bouillon did not grow.

In order to follow the growth of the ramifying threads and how they begin to form, some of the colonies were emulsified in sterile water and then plated; examination of slides made from young colonies showed that the organisms (micro-photographs of which are given in Plate II) were not separate ones but stages of transformation and further that the flagellated organism was not a separate one but a stage of the same organism.

Here then we are dealing with one organism with four stages which forms nitrites from ammonium sulphate but no nitrates.

Thermal Death Point.

As we have seen, this organism survives the temperature of 60°C while the nitrifiers described by Winogradsky and Omélianski do not survive 55°C. The maximum temperature which this organism will survive was found by inoculating separate flasks containing 50 c.c. dilute Omélianski solution, with a liquid culture diluted in sterile water (4 c.c. in 200 c.c.) by means of a sterile pipette, the same amount of inoculum, 5 c.c., being used; the flasks were then heated at different temperatures—*viz.*, 40°, 50°, 60°, 70°, 80°, 90°, and 100°C for half an hour.

Nitrites were estimated at the end of 6 weeks' incubation at 30°C, and gave the following results :—

	Milligrams of Nitrogen as Nitrite in 50 c. c. medium containing 5 mgms. Nitrogen as ammonium sulphate.
30°C	4·2
40°C	3·4
50°C	2·4
60°C	1·2
70°C	0·8
80°C	*Nil.*
90°C	*Nil.*
100°C	*Nil.*
Control (without any inoculum)	*Nil.*

Nitrates were not found in any of the flasks and so it need not be suspected that absence of nitrites may mean further oxidation to nitrates,

since nitrates were not found even after 8 weeks' growth in any of the flasks which shows that this organism forms nitrites only.

Effect of Temperature.

Liquid culture from the previous experiment was diluted and used as inoculum as in the previous experiment: after incubation at different temperatures for 4 weeks, quantitative tests for nitrites were made, duplicate flasks being used for each variation of temperature.

Incubated at	Milligrams N. as Nitrite.
20°C	2·4, 2·2
25°C	2·6, 2·8
30°C	3·0, 3·4
35°C	0·3, 0·7
40°C	*Nil.*, *Nil.*
Control without inoculum at 30°C	*Nil.*

Nitrates were not found at the end of the experiment in any of the flasks. Some flasks were incubated for a further period of 4 weeks but no nitrates were found. The optimum temperature for this organism lies between 25° and 35°C.

Effect of $MgCO_3$.

As noted previously, $MgCO_3$ was found to have an inhibiting influence on nitrification with higher concentration of ammonium sulphate when soil was used as inoculum. It was proposed to try whether the same holds good for this organism. The liquid culture (that incubated at 30°C) from previous experiment was used for inoculation as before, with varying concentration of ammonium sulphate.

The results after 4 weeks' incubation at 30°C are given below:—

	Milligrams N. as Nitrite.
Dilute Omélianski solution + $CaCO_3$	2·0, 1·9
Dilute Omélianski solution + $MgCO_3$	1·2, 1·0
Strong Omélianski solution + $CaCO_3$ (containing 4 times the amount of nitrogen as ammonium sulphate).	3·4, 3·6
Strong Omélianski solution + $MgCO_3$	1·2, 1·0

It will be seen from the above that $MgCO_3$ is much less effective than $CaCO_3$ as a base for nitrite formation in dilute as well as in strong solution.

Winogradsky[1], Godlewski[2] and Coleman[3] have shown that nitrifying organisms make use of CO_2 from air as their source of carbon. It was proposed to try the action of CO_2 by increasing its concentration in the atmosphere surrounding. Along with this the action of coal gas was also tried.

For this experiment duplicate flasks containing sterile Omélianski solution were inoculated from a liquid culture as before and kept under a stoppered glass bell-jar. This was partially exhausted of air under the pump with pressure gauge and when the latter indicated $\frac{1}{2}$ atmosphere the stopcock was closed and CO_2 was admitted till atmospheric pressure was reached; the stopcock was then closed again. The experiment was repeated with coal gas under a separate bell-jar.

Trial was also made as to whether the organism would grow without any oxygen. For this two flasks similarly inoculated as before were kept in a desiccator from which as much air was exhausted as possible and the oxygen from the remaining portion was absorbed by means of pyrogallic acid and caustic potash.

The results of the experiment show that CO_2 and coal gas have a stimulating effect on the organism. Under anaerobic conditions the organism does not grow. The amounts of nitrites formed are given below:—

	Milligrams N. as Nitrite in 50 c.c. Omélianski solution.
Without any treatment	1·2 1·4
CO_2	1·9 1·8
Coal gas	1·6 1·8
Anaerobically	*Nil.*
Control (without inoculation)	*Nil.*

In order to see whether the stimulating effect of CO_2 is due to some of it being dissolved in water thus rendering more $CaCO_3$ soluble and making the ammonium salt more easily accessible to the bacteria, another experiment was undertaken in which the Omélianski solution in which all insoluble

[1] *Annales de l'Inst. Pasteur T.* IV, 1890, p. 268.
[2] *Centr. für Bakt.* II, Abt. Bd. 2, 1896, p. 458.
[3] *Centr. für Bakt.* II, Abt. Bd. 20, 1908, p. 484.

$CaCO_3$ was filtered out before sterilization was compared with the unfiltered solution. The same (filtered and the unfiltered flasks) were kept in an atmosphere which contained 50 per cent. CO_2. For comparison flasks were kept in ordinary air. At the end of 4 weeks' incubation the results were as follows :—

	Milligrams N. as Nitrite. (Filtered solution.)		Milligrams N. as Nitrite. (Unfiltered solution.)
Under CO_2	0·85 0·89	Under CO_2	4·20 4·12
Without CO_2	0·60 0·68	Without CO_2	3·43 3·10

It is clear, therefore, that unfiltered flasks under CO_2 give better results. No nitrates were found.

To determine the effect of an increased proportion of CO_2, inoculated flasks were kept under a bell-jar and the air exhausted more or less completely by means of a pump and then CO_2 introduced into it and a comparison made with the ordinary atmosphere; the results at the end of 4 weeks were as follows :—

	Milligrams N. as Nitrite.
Ordinary atmosphere	2·05 2·39
CO_2 atmosphere	1·63 1·30

Various organic substances were found by Winogradsky and Omélianski to inhibit nitrification; it was proposed to try the action of glucose, asparagin and urea on this organism.

To separate flasks, each containing Omélianski solution (50 c.c. each), sterile solutions of the above substances were added by means of a sterile pipette. Inoculations were made as before by a liquid culture.

	Milligrams N. as Nitrite.
50 c.c. Omélianski solution + 0·1 gram Glucose	1·6 1·3
50 c.c. „ „ 0·2 „ Glucose	*Nil.* *Nil.*
50 c.c. „ „ + 0·1 „ Asparagin	4·1 3·8
50 c.c. „ „ + 0·2 „ Asparagin	2·8 2·5

	Milligrams N. as Nitrite.
50 c.c. Omélianski solution + 0·1 gram Urea ..	6·03 6·2
50 c.c. „ „ 0·2 „ Urea ..	4·1 4·3
50 c.c. without any addition ..	3·1 3·1

0·2 gm. glucose totally inhibits nitrite formation.
0·1 gm. glucose retards it strongly.
0·2 gm. asparagin retards it slightly.
0·1 gm. asparagin and 0·1 gm. and 0·2 gm. urea increase nitrite formation.

At the end of each experiment plates were made and did not show any contamination; in some of the experiments that follow platings were now and then made. There was no contamination in some cases, but sometimes colonies of other bacteria appeared; cultures were made from these organisms to see whether any of them when inoculated into Omélianski solution produced nitrites. None of the intruding organisms produced any nitrites or nitrates. This however does not exclude the possibility of their help through symbiosis with this organism; this objection could have been met if it had been possible to assert the absence of these intruding bacteria when they did not appear on the plates previously.

Unfortunately the appearance of one of these latter organisms is so watery when grown on silicate jelly and ammonia sulphate agar that it is quite possible that it might have escaped notice on these media. On other media none of the organisms appeared. Although as many precautions as were possible were taken, still in some of the plates contamination with these organisms did occur. If they prove to be inert organisms their appearance will not detract from the value of the experiments, but if they happen to be acting in some other way, which will be tested hereafter, some of the conclusions will have to be modified.

There were three kinds of intruding organisms, all of which grew on ordinary agar + 5°F.

(1) Watery growth .. Small rod-shaped. This was the most frequent contamination.
(2) Yellow growth .. Small rod-shaped.
(3) Brown .. Small rod-shaped.

The fact that these three organisms alone appeared whenever any contamination occurred, and the fact that organisms of chance contamination in

this laboratory are of quite a different type excluded the possibility of these organisms being chance contaminations and leads to the alternative suppositions either that they must have some specific natural association with the organism under examination or that their growth is favoured by the cultural conditions of the experiments described.

In order to see which source of nitrogen is preferred by the organism described in this paper, solutions were prepared in which asparagin, urea, NH_4Cl and $(NH_4)_2CO_3$ replaced $(NH_4)_2SO_4$ in the culture solution. It was also proposed to see which of these required $CaCO_3$ as a base for neutralizing the acid. These were then sterilized and inoculated with an equal amount of inoculum :—

	Milligrams N. as Nitrite.
Asparagin without $CaCO_3$ ..	.. { 0·84 0·80
Asparagin with $CaCO_3$	.. { 1·40 1·20
Urea without $CaCO_3$	.. { 1·70 1·60
Urea with $CaCO_3$	.. { 0·03 0·03
$(NH_4)_2CO_3$ without $CaCO_3$	.. { 1·90 2·00
$(NH_4)_2CO_3$ with $CaCO_3$	.. { 0·24 0·24
NH_4Cl without $CaCO_3$	.. { 0·25 0·20
NH_4Cl with $CaCO_3$	.. { 1·90 2·00
$(NH_4)_2SO_4$ without $CaCO_3$	.. { 0·40 0·40
$(NH_4)_2SO_4$ with $CaCO_3$	.. { 1·60 1·80

It is interesting to note that $CaCO_3$ has an inhibitory action with $(NH_4)_2CO_3$ and urea, while in other cases it is favourable. Probably the $(NH_4)_2CO_3$ and urea solutions are by themselves sufficient to supply the most easily available source of food to the nitrite former and addition of $CaCO_3$ which is so necessary in other cases is not useful in the case of the two substances noted, and probably some of the ammonia might be lost by volatilization after the addition of $CaCO_3$. Nitrates were not found in any of the flasks, which excludes the possibility of lowering of nitrite on account of further oxidation.

In the next experiment 0·05 per cent. solution of asparagin, urea, $(NH_4)_2 CO_3$, $NH_4 Cl$, $(NH_4)_2 SO_4$ were used with and without $CaCO_3$ to see whether the nitrite-former can utilize them in a solution containing no phosphate. Results after 4 weeks' incubation are given in the following table :—

	Milligrams N. as Nitrite.
Asparagin solution	*Nil.* *Nil.*
Asparagin solution + $CaCO_3$	*Nil.* *Nil.*
Urea solution	0·07 0·06
Urea solution + $CaCO_3$	0·02 0·02
$(NH_4)_2 CO_3$ solution	1·20 1·00
$(NH_4)_2 CO_3$ solution + $CaCO_3$	0·24 0·25
$NH_4 Cl$ solution	0·02 0·02
$NH_4 Cl$ solution + $CaCO_3$	0·03 0·03
$(NH_4)_2 SO_4$ solution	0·01 0·01
$(NH_4)_2 SO_4$ solution + $CaCO_3$	0·05 0·04

The inhibitory effect of $CaCO_3$ on nitrite formation is noticeable in the case of $(NH_4)_2 CO_3$ and urea.

That $CaCO_3$ when added to other salts gives more nitrites confirms Ashby's[1] supposition that the other salts require to be converted to $(NH_4)_2 CO_3$ for the nitrite-former to turn them into nitrite.

SUMMARY.

1. The nitrite-forming organism described in this paper is a new one, differing morphologically from others hitherto known.

2. Its thermal death point lies between 70°C and 80°C.

3. The optimum temperature for its activity is between 25°C and 35°C.

4. $MgCO_3$ is much less effective than $CaCO_3$ as a base for nitrite formation by this organism.

[1] Ashby, *Journ. of Agrl. Science*, Vol. II, 1902.

5. (*a*) An increased proportion of CO_2 in the atmosphere (50 parts per 100) acts as a stimulus to the activity of this organism ; further increase of CO_2 has a retarding effect.

(*b*) Coal gas exercises a similar influence.

6. 0·2 gm. glucose in 50 c.c. Omélianski solution totally inhibits nitrite formation by this organism.

0·1 gm. glucose in 50 c.c. Omélianski solution retards it strongly.

0·2 gm. asparagin in 50 c.c. Omélianski solution also retards it.

0·1 gm. asparagin and 0·1 gm. urea and 0·2 gm. urea in 50 c.c. Omélianski solution shows a rise in nitrite formation by this organism.

7. (*a*) In solutions containing phosphates, ammonium chloride, ammonium sulphate, ammonium carbonate, asparagin and urea serve as sources of nitrogen for nitrite formation.

(*b*) In the case of $(NH_4)_2\ CO_3$ and urea, $CaCO_3$ appears to exert an inhibitory influence.

8. In solutions containing no phosphates, $(NH_4)_2\ CO_3$ is the only substance which is easily changed into nitrites.

9. The presence of protozoa along with nitrite-formers might easily have escaped notice, had not the use of filter paper pads in cultural technique favoured their development.

PUSA,
November 29th, 1914.

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